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A Sensitive Cyclic Nucleotide Phosphodiesterase Assay for Transient Enzyme Kinetics

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A new assay for cyclic nucleotide phosphodiesterase has been developed by using reverse-phase column chromatography for the separation of product and substrate of the enzymatic reaction. The polar 5′-nucleotides are not retarded by the column, while the more lipophilic cyclic nucleotides bind to the column. Properties such as pH and ionic strength of the incubation mixture or the elution buffer have only minor effects on the elution pattern. The assay by reverse-phase chromatography has several advantages above other assay methods currently employed; it is fast and simple, has a very low blank (0.2%), and is very sensitive (1 fmol). The assay can be used for different substrates (cyclic AMP, cyclic GMP, cyclic IMP) without modification of the conditions. The usefulness of the assay is demonstrated by transient kinetic measurements on a time scale in seconds of a cGMP-dependent cGMP-specific phosphodiesterase from the cellular slime mold *Dictyostelium discoideum*.

Cyclic nucleotides play a key role in the regulation of metabolism, function, and growth of many cell types (1). The regulation of cyclic nucleotide levels in biological systems is mediated by nucleoside triphosphate pyrophosphate-lyase (i.e., adenylate cyclase or guanylate cyclase) and by 3′,5′-cyclic nucleotide 5′-nucleotidohydrolase (e.g., cAMP[2]-phosphodiesterases). Since cyclic nucleotide levels may change within a few seconds after hormonal stimulation of sensitive cells, analysis of phosphodiesterase activity during the first seconds of the incubation *in vitro* may help to understand the function of the enzyme *in vivo*. Such an assay requires that very small amounts of product (femtomoles) are separated from a large bulk of substrate. Previously described assays (for review see (2)) lack high sensitivity, produce too high blanks, or are time consuming and, therefore, inconvenient for a large number of samples. One assay, making use of activated magnesium silicate (3), is simple, sensitive, and accurate, but is only applicable to cAMP hydrolysis.

In this paper a simple method is described for the separation of small amounts of 5′-nucleotides from large amounts of cyclic nucleotides. The high resolution of the assay is demonstrated by experiments on the transient kinetics of a cGMP-stimulated phosphodiesterase.

MATERIALS AND METHODS

Chemicals. cIMP and 8-bromoguanosine 3′,5′-monophosphate (c^{8}b-GMP) were purchased from Boehringer, Mannheim, Germany; reverse-phase packing material (μBondapak C_{18}/Porasil B, 35–75 μm) was obtained from Waters Associates Inc., Milford, Massachusetts; [8-^{3}H]cGMP (0.55 TBq/mmol) and [8-^{3}H]cAMP (0.95 TBq/mmol) were obtained from the Radiochemical Centre, Amersham, England. [8-^{3}H]cIMP was syn-

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[2] Abbreviations used: cAMP, adenosine 3′,5′-monophosphate; cGMP, guanosine 3′,5′-monophosphate; cIMP, inosine 3′,5′-monophosphate; c^{8}b-GMP, 8-bromoguanosine 3′,5′-monophosphate; 10 Pb 7, 10 mM Sørensen phosphate buffer, pH 7.0.

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thesized from [8-^{3}H]cAMP by the method of Casnelli *et al.* (4), with the exception that pyridine acetate was replaced by ammonium acetate during the purification. The product was stored in 50% (v/v) aqueous ethanol at a concentration of 10^{-5} M. Radioactive 5'-nucleotides were synthesized from their radioactive cyclic nucleotides by incubation with phosphodiesterase and purified on a reverse-phase column (0.5 ml, pH 7.0). Radioactive nucleosides were prepared by further degradation with 5'-nucleotidase. All chemicals used were of analytical grade.

Preparation of reverse-phase column. A 6 × 18-mm (0.5 ml) and a 6 × 11-mm (0.3 ml) reverse-phase column were prepared by pouring a suspension of reverse-phase material in 50% (v/v) aqueous methanol into short Pasteur capillary pipets plugged with a small piece of glass wool. Unless otherwise stated, the columns were washed with 3 ml elution buffer (phosphate buffer, containing 1% (v/v) methanol). The columns were regenerated by washing them with 3 ml regeneration buffer (phosphate buffer containing 50% (v/v) methanol), after which the columns were washed with 3 ml elution buffer.

Purification of the substrate. Immediately before use in the assay, the [8-^{3}H]cGMP was purified to above 99.9% on the 0.5-ml reverse-phase column. [8-^{3}H]cGMP (2.5 μl stock solution) was diluted to 200 μl with 10 mM Sørensen phosphate buffer, pH 7.0 (10 Pb 7), and applied to the column. The column was then washed at atmospheric pressure with 1 ml 1% methanol, 10 Pb 7. [8-^{3}H]cGMP was eluted with 5% methanol, 10 Pb 7, and the radioactive fractions were pooled. [8-^{3}H]cIMP was purified in the same way. [8-^{3}H]cAMP did not elute with 5% methanol, due to its greater hydrophobicity. Therefore, [8-^{3}H]cAMP was eluted with 1 ml 10% methanol, 10 Pb 7.

Phosphodiesterase assay. cGMP-dependent cGMP-specific phosphodiesterase was partially purified from *Dictyostelium discoideum* NC-4(H) as described previously (5). The incubation mixture had a total volume of 150 μl and contained 10 mM phosphate buffer, pH 7.2, 10^{-7} M [8-^{3}H]cIMP (about 1 kBq), different concentrations of c^{8}b-GMP, and cGMP-phosphodiesterase (0.085 mg protein derived from 2×10^7 cells). The reaction was initiated by adding enzyme to the substrate, while mixing vigorously on an Eppendorf mixer 5432. The reaction was terminated by the addition of 100 μl ice-cold 20 mM phosphoric acid, which reduced the pH to 3.0.

Part of the incubation mixture (200 μl) was applied to the 0.3-ml reverse-phase column, equilibrated with 10 mM KH_2PO_4/H_3PO_4, pH 3.0 (10 Pb 3), 1% methanol. The product was eluted with 1 ml of the same buffer. The cyclic nucleotide was subsequently eluted with 1.2 ml 10 Pb 3, 50% methanol. To the different fractions 1.5 ml Instagel (Packard) scintillation solvent was added, and the radioactivity was determined with a LKB liquid scintillation counter.

RESULTS AND DISCUSSION

Chromatography of cGMP and 5'-GMP by Reverse Phase

5'-GMP is only slightly retarded by a reverse-phase column, and coelutes with the first four column volumes of elution buffer (10 Pb 3, 1% methanol). cGMP is retarded more strongly, and elutes as a broad peak (Fig. 1a). On a reverse-phase system retardation of solutes can be reduced by decreasing the polarity of the buffer with methanol (6). Elution of cGMP is very much enhanced with 10 Pb 3, 50% methanol. Using a two-step elution with, respectively, 1 and 50% methanol, a rapid and complete separation of 5'-GMP and cGMP is achieved (Fig. 1b). In this separation system all 5'-nucleotides (5'-AMP, 5'-GMP, and 5'-IMP) elute in fraction A, while the cyclic nucleotides and the nucleosides elute in fraction B.

Effects of pH and Ionic Strength of the Buffers on the Elution Pattern

Protonation of a basic group of a solute results in an increase of the polarity leading to an accelerated elution from reverse-phase columns. Protonation of an acidic group leads

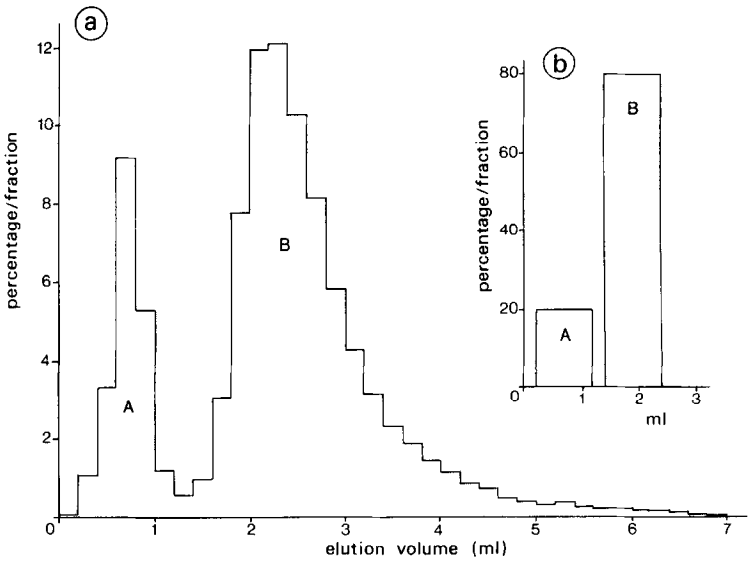

FIG. 1. Separation of 5'-GMP from cGMP by reverse-phase chromatography. A mixture (200 μl) of 20% [8-^{3}H]5'-GMP and 80% [8-^{3}H]cGMP was applied to a 0.3-ml reverse-phase column. (a) The column was eluted with 10 Pb 3, 1% methanol. The radioactivity in 200-μl fractions was determined. Peak A is 5'-GMP and peak B is cGMP. (b) 5'-GMP was eluted with 1 ml 10 Pb 3, 1% methanol (fraction A). cGMP was subsequently eluted with 1.2 ml 10 Pb 3, 50% methanol. The first 200 μl of this fraction was collected separately to check for complete separation. The other 1 ml eluant contained all the cGMP (fraction B).

to a decrease of polarity, and thus to increased retention by reverse-phase columns. Therefore, the elution pattern of solutes is modified if the pH is changed around a pK value of the solutes (6). The elution pattern of 5'-GMP and cGMP is not changed between pH 3.0 and 6.0 in the two-step separation system (Fig. 1b), due to the absence of a pK value in this pH range.

Previously it has been shown that low concentrations of ions in the elution buffer (0–1 mM) may change the elution pattern of charged solutes (7). It has also been shown that higher ion concentrations (above 1 mM) have only minor effects on the elution pattern (6). In accordance with these observations, the elution patterns of 5'-GMP and cGMP are not changed by the addition of sodium chloride (0–1 M) to the elution buffer, which contained 10 mM sodium-potassium phosphate.

Avoidance of the Effect of 5'-Nucleotidase Impurities

Accurate measurements on the amount of substrate hydrolyzed by the assay in Fig. 1b is not possible when 5'-GMP is subsequently degraded to guanosine, since cGMP and guanosine coelute. Impurities of 5'-nucleotidases disturb the assay in the same way if cAMP or cIMP are used as the substrates of the phosphodiesterase reaction. The effects of 5'-nucleotidase impurities are avoided by coelution of guanosine and 5'-GMP. The procedure is as follows: charged solutes tend to form ion pairs with oppositely charged buffer ions. By increasing the hydrophobicity of the positively charged buffer ions 5'-GMP and cGMP—which both have a negative charge—will become more hydrophobic, and are retarded more strongly by reverse-phase columns. The degree of retardation depends on the ratio of concentrations of polar and hydrophobic cations in the elution buffer (6). The elution of guanosine—which has no negative charge—is not affected by the hydrophobicity of the buffer ions. By addition of the correct amount of tributylammonium to the elution buffer 5'-GMP is retarded to such an extent that it coelutes with guanosine. Guanosine and 5'-GMP are eluted in the first fraction by increasing the methanol concentration from 1 to 10%.

The optimal conditions are elution of guanosine and 5'-GMP with 1 ml 8 mM tributylammonium phosphate, 10 mM phosphate buffer, pH 3.0, 10% methanol, followed by the elution of cGMP with 1.2 ml 10 mM phosphate buffer, pH 3.0, 50% methanol. The procedure to avoid the effect of 5'-nucleotidase impurities with cAMP or cIMP as the substrate is identical to the method described above.

Assay Conditions

For high sensitivity of the assay very low background counts are required. To enhance this, the radiolabeled substrate, which is susceptible to radiolytic decomposition, was purified to above 99.9% immediately before use in the phosphodiesterase assay on the 0.5-ml reverse-phase column (Fig. 2). The elution of substrate with 5% methanol resulted in a final methanol concentration of 0.83% (v/v) in the assay. Measurement of the effect of methanol on the enzyme activity showed that the activity is not affected by 1% methanol (v/v) (data not shown).

The use of ice-cold phosphoric acid resulted in instantaneous termination of the reaction. For other kinds of enzyme this termination method is not always rigorous enough (unpublished data). As an alternative method the enzyme reaction is terminated by the addition of 1.5% (v/v) aqueous $HClO_4$ followed by adjusting the pH to 3.0. However, this method results in a slight increase of the blank of the assay. Termination by boiling is less instantaneous and gives rise to chemical breakdown of substrate (cGMP about 1.6%/min, cAMP about 0.5%/min).

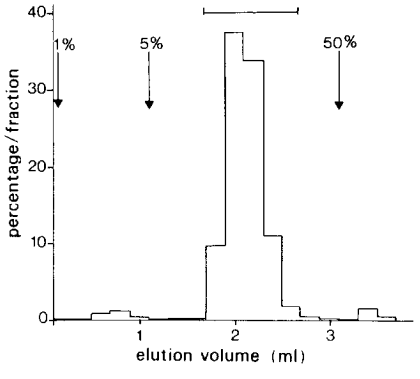

FIG. 2. Purification of substrate by reverse-phase chromatography. Two hundred microliters [8-^{3}H]cGMP containing about 0.1 MBq was applied to a 0.5-ml reverse-phase column. Radioactive water and [8-^{3}H]5'-GMP were eluted with 10 Pb 7, 1% methanol, and [8-^{3}H]cGMP was eluted with 10 Pb 7, 5% methanol. The five fractions that contained the largest amount of radioactivity were pooled and used in the phosphodiesterase assay. Additional impurities are eluted with 50% methanol.

TABLE 1

BLANK AND REPRODUCIBILITY

	Fraction A (cpm)	Fraction B (cpm)
Mobile phase[a]	11 ± 1	10 ± 2
Blank[b]		
[^{3}H]cAMP	49 ± 7	19,898 ± 726
[^{3}H]cGMP	42 ± 8	21,161 ± 457
[^{3}H]cIMP	33 ± 5	15,705 ± 340
Incubation[c]	462 ± 29	15,326 ± 470

Note. All results shown are the means ± SD of nine measurements.

[a] Counts per minute of the mobile phase were measured by determining radioactivity of 1 ml mobile phase in 1.5 ml scintillation fluid.

[b] The blank was determined by adding 100 μl ice-cold 20 mM phosphoric acid to the substrate, before adding the enzyme.

[c] Ten-second incubation of [8-^{3}H]cIMP with c^{8}b-GMP preactivated enzyme (see Fig. 3).

The purification of substrate and the mild, but instantaneous termination result in a very low blank and a high reproducibility (Table 1). The blank of 40 cpm represents about 4 fmol product.

The purity and identity of the radioactivity in fractions A and B was determined by high-performance liquid chromatography which separates nucleosides, 5'-nucleotides, and cyclic nucleotides. The radioactivity of fraction B (blank and incubation) was identified as at least 99% cyclic nucleotides; small amounts (0.1–0.3%) were nucleosides and a small amount (about 0.5%) appeared in the void volume of the column (the identity is probably ^3H_2O). The radioactivity of the blank in fraction A was distributed all over the chromatogram, with the majority appearing in the void

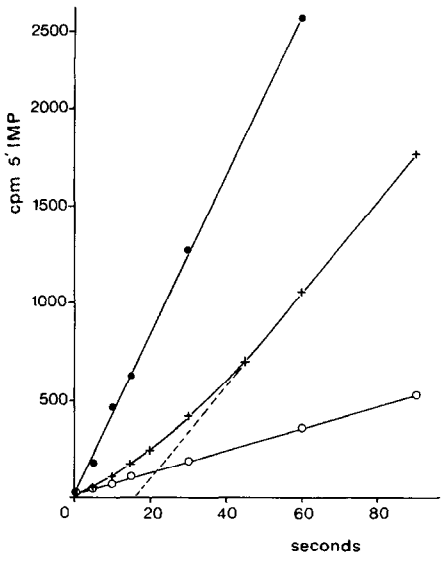

FIG. 3. Transient kinetics of the hydrolysis of [8-^{3}H]cIMP by the cGMP-dependent phosphodiesterase isolated from the cellular slime mold *D. discoideum* (5). The enzyme was rapidly mixed with 0.1 μM [8-^{3}H]cIMP (containing about 20,000 cpm). The reactions were terminated at the times indicated, and the reaction mixture was chromatographed on 0.3-ml reverse-phase columns. O, [^{3}H]5'-IMP formed after mixing nonactivated enzyme with [^{3}H]cIMP. ●, The enzyme was preincubated with 0.5 μM c^{8}b-GMP for 5 min; at t = 0 the activated enzyme was mixed with [^{3}H]cIMP and 0.5 μM c^{8}b-GMP. +, The enzyme was not preactivated by c^{8}b-GMP; however, activation was started at t = 0 by mixing the nonactivated enzyme with [^{3}H]cIMP and 0.1 μM c^{8}b-GMP.

volume. The radioactivity of incubation in fraction A consists mainly of 5'-IMP (at least 90%) and a small amount of radioactivity eluted in the void volume.

Transient Kinetics of cGMP-Dependent cGMP-Specific Phosphodiesterase

The usefulness of the present assay is demonstrated with a cGMP-stimulated phosphodiesterase isolated from the cellular slime mold *D. discoideum*. cIMP is an analog specific for the catalytic site, while 8-bromoguanosine 3',5'-monophosphate is an analog specific for the activator site. The results of Fig. 3 reveal that a steady state is reached at the catalytic site within 5 s, while activation of the enzyme is slower, reaching a steady state only after 30–45 s. The present assay is sufficiently sensitive and accurate to allow mathematical processing of the data of the hydrolysis of cIMP during the first seconds of the incubation (8).

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REFERENCES

1. Robison, G. A., Butcher, R. W., and Sutherland, E. W. (1971) Cyclic AMP, Academic Press, New York.
2. Thompson, W. J., Terasaki, W. L., Epstein, P. M., and Strada, S. J. (1979) *Adv. Cyclic Nucleotide Res.* **10**, 69–92.
3. Sinha, A. K., and Colman, R. W. (1981) *Anal. Biochem.* **113**, 239–245.
4. Casnelli, J. E., Ives, H. E., Jasmieson, J. D., and Greengard, P. (1980) *J. Biol. Chem.* **255**, 3770–3776.
5. Bulgakov, R., and Van Haastert, P. J. M. (1983) *Biochim. Biophys. Acta* **756**, 56–66.
6. Van Haastert, P. J. M. (1981) *J. Chromatogr.* **210**, 229–240.
7. Van Haastert, P. J. M., De Wit, R. J. W., Grijpma, Y., and Konijn, T. M. (1982) *Proc. Nat. Acad. Sci. USA* **79**, 6260–6274.
8. Van Haastert, P. J. M., and Van Lookeren Campagne, M. M. (1982) *J. Cell Biol.*, in press.